Breaking the diffraction limit for label-free chemical imaging

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Super-resolution microscopy has long been a cornerstone in the study of molecular biology, allowing scientists to observe processes at the nanoscale. However, most of these techniques rely on fluorescent labels, which not only require complex preparation but also pose limitations in imaging certain small molecules.[1] Stimulated Raman scattering (SRS) is a label-free imaging technique that offers chemical selectivity by capturing the intrinsic vibrations of intracellular biomolecules.[2] However, the resolution limit of a laser-scanning SRS system is at ~300 nm, which is insufficient for capturing the subcellular nanostructures inside the cells. How to achieve label-free super-resolution imaging of biological samples remains a heated topic in the field.

In SRS microscopy, two fields, namely pump and Stokes, are tightly focused on the sample to excite the nonlinear Raman process. Such a process induces an energy change in the original laser fields, which can be extracted through a lock-in amplifier. The SRS signal, generated in regions where the two fields overlap, offers a resolution roughly equivalent to that of two-photon imaging, providing a $\sqrt{2}$-fold enhancement over the one-photon imaging.

Structured illumination microscopy (SIM) is well-known for breaking the diffraction limit by a factor of two.[3] By incorporating structured illumination into SRS, the resolution could theoretically improve by a factor of $\sqrt{2}$, shifting from $\frac{0.61\lambda}{\sqrt{2}\mathrm{NA}}$ to $\frac{0.61\lambda}{2\mathrm{NA}}$. Recently published in *Advanced Imaging*,[4] Hilton B. de Aguiar and his colleagues from the Kastler-Brossel Laboratory in France present Blind-S3, a super-resolution technique that successfully translates this concept into reality.

Unlike conventional wide-field SIM, the authors implemented a hybrid illumination design. They used speckle illumination for the pump beam and laser-scanned the Stokes beam to capture the SRS image with a single-element detector. A series of speckle-illuminated images were then reconstructed using advanced computational methods. This synergistic "instrumentation + computation" approach not only overcomes the challenge of SRS signal detection without a camera but also enhances the penetration depth of SRS, thanks to the use of speckle illumination. Blind-S3 achieves a $\sqrt{2}$-fold resolution enhancement, as demonstrated through imaging of HeLa cells and thick mouse brain tissues.

Blind-S3 now joins the ranks of super-resolution, label-free Raman imaging technologies, with the ultimate goal of achieving nanoscopy of non-fluorescent species in biological samples. Other super-resolution techniques rely on mechanisms such as higher-order susceptibility,[5] molecular saturation,[6,7] stimulated emission depletion,[8] photoswitching,[9,10] or sample expansion.[11,12] However, these methods often require non-biocompatible high laser powers, fluorescent labeling (which reintroduces the challenges of fluorescence compatibility), or special sample treatments. In contrast, Blind-S3 shows great promise for broader application in natural, unprocessed biological samples.

Despite its significant advancements, the current resolution of Blind-S3 is still distant from the capabilities of fluorescent nanoscopy. This limitation stems not only from the near-infrared excitation, which offers lower fundamental resolution, but also from insufficient sensitivity to detect minute nanostructures. Thus, the quest to develop novel vibrational imaging techniques that can achieve sub-100 nm resolution while maintaining the sensitivity to detect non-fluorescent metabolites remains an ongoing and intense area of research.

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